SYMBIOGENETICS

Some aspects of metal ion transport and *in silico* gene expression analysis of potassium/sodium ion transporters, channels and exchangers in root nodules

Natalia Trifonova, Maria Koroleva, and Elena Fedorova

K. A. Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, ul. Botanicheskaya, 35, Moscow, 127276, Russian Federation

Address correspondence and requests for materials to Elena Fedorova, elenafedorova06@mail.ru

Abstract

Rhizobia establish a symbiotic relationship with legumes, which results in the formation of root nodules, the ecological niche for intracellular rhizobia. The infected cell of a root nodule is a special integral unit of plant and nitrogen fixing rhizobia. Nodules tend to be very sensitive to ionic stresses, such as salt stress. High vulnerability toward ionic stresses might be due to defects in ion balance and transport in the infected tissue. The purpose of this minireview is to summarize the current data regarding metal ion transport in the root nodule, with particular emphasis on potassium/sodium ion transport. A bioinformatic approach and *in silico* gene expression analysis have been used to obtain some insight for K^+/Na^+ transporter channels and exchangers in root nodule developmental zones.

Keywords: symbiosis, root nodule, infected cell, symbiosome, ion transporters

Introduction

Soil bacteria from the family Rhizobiaceae are able to form a symbiotic relationship with leguminous plants. Symbiosis gives plants access to ammonia converted from the nitrogen gas by bacterial enzyme nitrogenase. The initial steps of symbiosis consist of signal exchange between two potential partners. Rhizobia recognize the flavonoids, secreted by legumes, and the Nod factors produced by the bacteria trigger a signal transduction pathway in the root cells that activates root nodule organogenesis (Oldroyd, 2013; Geurts et al., 2016; Roy et al., 2020).

Completely new organs, the root nodules, are formed by a new meristem, which is initiated by the dedifferentiation of root cortical cells. The spatial pattern of meristem initiation and persistence determines the main differences between nodule types. Nodules of so-called indeterminate type of growth (*Medicago truncatula, Pisum sativum*) develop as elongated cylindrical structures due to the meristem situated on the nodule apex, and the nodules with determinate type of growth (*Lotus japonicus*, *Glycine max*) develop in spherical form due to the short living meristem situated in the centre of the primordia (Vasse et al., 1989; Kondorosiet et al., 2013; Mergaert et al.,2020).

Rhizobia start to populate the host cells' apoplast, forming small local colonies, and in the next stage of infection they invade the symplastic space of the host cells via infection threads in the so-called zone of infection (Vasse et al., 1990). Infected cells undergo polyploidization, enlarge, and differentiate into fully matured symbiotic cells that contain nitrogen-fixing bacteroids (Kondorosiet al., 2013; Mergaert et al., 2020). The zone of the nodule, composed of mature infected cells, is defined as the zone of nitrogen fixation. The lifespan of an infected cell is around 16–20 days before the symbiotic relations are terminated, nitrogen fix-

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Authors' information: Natalia Trifonova, PhD Student, Engineer, orcid.org/0000-0002-3493-9994; Maria Koroleva, Master Student, Engineer, orcid.org/0000-0001-9920-7383; Elena Fedorova, PhD, Leading Researcher, orcid.org/0000-0003-3953-1282

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ation ceases, and the cell undergoes lysis. For information concerning the morphology and organogenesis of root nodules we would like to refer to excellent reviews published recently (Oldroid et al., 2013; Kondorosiet al., 2013; Mergaert et al., 2020).

About half of the cells of the nodule inner cortex contain rhizobia, hence are infected. The intracellular form of bacteria, the symbiosome — or nitrogen-fixing "organelle" — is a host plant-derived membrane vesicle housing single or several bacteria (Parniske, 2018; Coba et al., 2019; Roy et al., 2020). Thousands of symbiosomes are kept within the infected cell's symplast, separated from the host cytoplasm by a host cell-derived membrane. This interface, the symbiosome membrane, is the key element of the symbiotic relationship. The transport of molecules and ions necessary for bacteria metabolism through the symbiosome membrane ensures the intracellular lifestyle of rhizobia and the functional activity of root nodules. Mature symbiosomes appear to have a unique mosaic identity, combining plasma membrane and late endosomal markers due to the retargeting of some host cell proteins toward the symbiosome membrane (Limpens et al., 2009; Ivanov et al., 2012; Gavrin et al., 2016, 2017). The infected cell is key to the maintenance of symbiotic interactions; the unique biology of this special integral unit of plant and bacteria justifies the study of the infected cell's development and special features.

One of the important factors in the development of an infected cell is ion homeostasis. The data obtained in recent years in this area have opened an unexplored field of research. However, it turns out that integral analysis of data obtained by different methods like genomics, cell biology and plant physiology is needed. The purpose of this minireview is to summarize the current data regarding metal ion transport in the root nodule with an emphasis on potassium/sodium ion transport, and to outline putative future research in this area. In the first part of this minireview we will briefly summarize the recent data concerning the host plant transporters of metal ions required for metalloenzymes, cofactors of key proteins involved in the process of nitrogen fixation, oxygen balance in the nodule and other functions. In the second part of the minireview we will focus on the putative channels and transporters of potassium and sodium in nodule developmental zones.

Ions required for the functional activity of infected cells in the root nodule

Infected cells of the root nodule have a distinct requirement for a number of ions. The process of nitrogen fixation is based on assembly and functionality of several metalloproteins. Specialized metalloenzymes and proteins are involved in the enzymatic reaction of nitrogen fixation and the maintenance of oxygen level in infected cells. These genes and metalloproteins have been intensively studied and reviewed in recent years (Rubio and Ludden, 2008; Brear et al., 2013; Mendoza-Suárez et al., 2020; Roy et al., 2020). Several metal ions are cofactors of metalloenzymes such as (Fe)-hemoglobins, (Fe-Mo)-nitrogenase, (Fe-S)-nitrogenase, (Cu-Fe)-cytochromes, and transcription factors including zinc finger (Roy et al., 2020). The search for the specific transporters by methods of forward and reverse genomics have been performed with the aim of understanding how these ions are transported to the nodule (Brear et al., 2013, 2020; Roy et al., 2020). Below we briefly describe the recent publication regarding ion transporters in the root nodule and infected cell. A schematic diagram of localization of transporters is given in Figure 1.

Molybdenum is the ion crucial for nitrogenase, a two-component metalloenzyme, that has molybdenum in the iron-molybdenum cofactor, the active center of the enzyme. Members of the molybdate transporter family Molybdate Transporter type 1 (MOT1) were identified in *M. truncatula* (Tejada-Jimenez et al., 2017). Expression analysis, yeast toxicity assays, confocal microscopy, and phenotypical characterization of Transposable Element from *Nicotiana tabacum* (Tnt1) insertional mutant line have been carried out in MtMOT1.3. Among the five MOT1 members present in the *M. truncatula* genome, MtMOT1.3 is the only one uniquely expressed in nodules. MtMOT1.3 shows molybdate transport capabilities when expressed in yeast. Immunolocalization studies revealed that MtMOT1.3 is located in the plasma membrane of nodule cells. A mot1.3-1 knockout mutant showed impaired growth concomitant with a reduction of nitrogenase activity. This phenotype was rescued by increasing molybdate concentrations in the nutritive solution, or upon addition of an assimilable nitrogen source. Furthermore, mot1.3-1 mutant plants transformed with a functional copy of MtMOT1.3 have shown a wild-type-like phenotype. These data are consistent with a model in which MtMOT1.3 is responsible for introducing molybdate into nodule cells, which is later used to synthesize functional nitrogenase (Tejada-Jimenez, 2017). In a recent study Gil-Díezetal et al. (2019) performed research on MtMOT1.2, another molybdenum transporter of this family. They characterized the function of MtMOT1.2 as a likely candidate for molybdate uptake from the vasculature by endodermal cells (Gil-Díezetal et al., 2019). MtMOT1.2 is located in the endodermis of the nodule and root vascular cylinders, in the plasma membrane and in an endomembrane compartment. It has shown molybdate uptake capabilities in yeast, and its mutation in *M. truncatula* leads to a reduction in the nitrogenase activity of nodules; likely it is the result of the reduction in molybdate delivery to the infected cells. The function of MtMOT1.2 seems to be relevant for symbiotic nitrogen

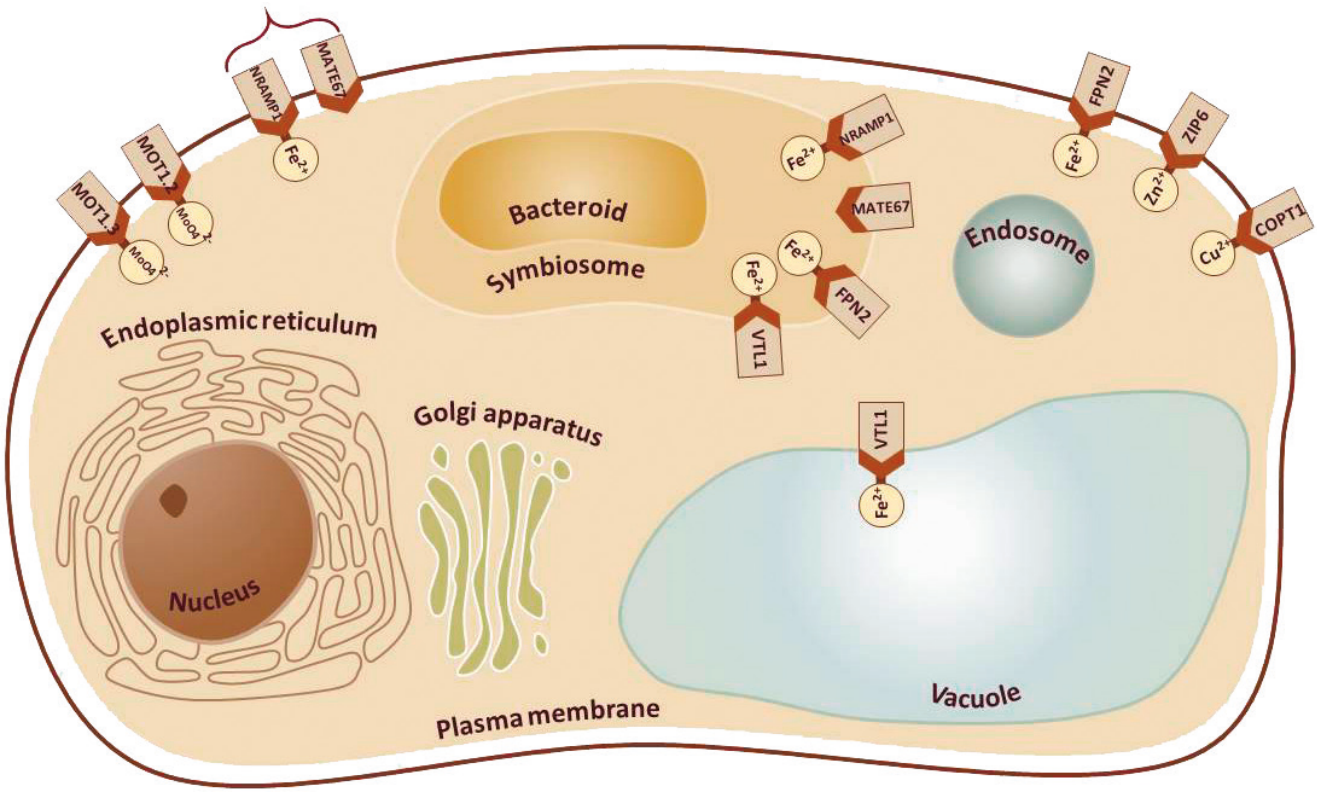

Fig. 1. The location of metal ion transporters involved in the enzymatic reaction of nitrogen fixation and the maintenance of oxygen level in infected cells.

COP1, **Copper** membrane transporter; **FPN2**, Ferroportin2, $Fe2^{+}$ translocator; **MATE67**, organic chelator, citrate efflux transporter; **MOT1-2,** Molybdate Transporter type 1; **MOT1-3,** Molybdate Transporter type 1; **NRAMP1**, Resistance-Associated Macrophage Protein1, $Fe2^{+}$ translocator; **ZIP6**, zinc transporterMtZIP6 (Zinc-IronPermease6); **VTL1,**Vacuolar Iron Transporter1.

fixation, given the fact that its mutation has no effect on plants grown on nitrate.

It can be assumed that molybdenum transporters MtMOT1.2 and MtMOT1.3 are essential for symbiotic nitrogen fixation (Fig. 1). However, the localization of these transporters in the symbiosome membrane has not yet been proven.

Copper membrane transporters of the COPT family have been identified by Senovilla et al. (2018) in *M. truncatula*. MtCOPT1 has been found to be the only nodule-specific COPT gene from the group of eight COPT transporters. Senovilla et al. (2018) performed phenotypical characterization of COPT1 Tnt1 insertional mutant, yeast complementation assays, expression analysis and confocal microscopy. MtCOPT1 has been located in the plasma membrane in young root nodule cells up to the early fixation zone. Mutation of MtCOPT1 results in diminished nitrogenase activity in nodules. Senovilla et al. (2018) have pointed out that such consequences can be a secondary effect due to the loss of a Cu-dependent function of cytochrome oxidase activity in bacteroids. Such effects result in the reduction of biomass production when the plant obtains its nitrogen exclusively from symbiotic nitrogen fixation, which proves the specific requirement of this ion for the symbiosomes (Fig. 1).

Iron. Iron deficiency is a nutritional problem in plants which reduces productivity and growth. Deficiency in iron can affect initiation and development of the nodule (Brear et al., 2013, 2020; Krivoruchko et al., 2018; Roy et al., 2020). Iron is required for iron- and molybdenum-iron proteins of nitrogenase, leggemoglobin, respiratory oxidases, and is essential for some enzymes of the bacterial respiratory chain. According to the data of Rodríguez-Haas et al. (2013), obtained by the methods of metal visualization and synchrotron-based x-ray fluorescence studies, most of the iron is delivered via vascular bundles to the apoplast of the zone of infection. In the zone of active nitrogen fixation, iron is mostly localized in symbiosomes (Rodríguez-Haas et al., 2013). In their interesting work, Escudero et al. (2020) have shown that Ferroportin2 (MtFPN2) is able to mediate iron import into symbiosomes in *M. truncatula* nodules. MtFPN2 is located in intracellular membranes in the nodule vasculature and in inner nodule tissues, as well as in the symbiosome membranes in the interzone and early-fixation zone of the nodules. Loss of function of MtFPN2 alters iron distribution in nodules, reducing nitrogenase activity and biomass production. Using promoters with different tissue activity to drive MtFPN2 expression in MtFPN2 mutants, Escudero et al. (2020) de-

termined that expression in the inner nodule tissues is sufficient to restore the phenotype, while confining that MtFPN2 expression to the vasculature did not improve the mutant phenotype.

The other plasma membrane iron transporter that transports iron from the apoplast into infected nodule cells is Resistance-Associated Macrophage Protein1 (MtNRAMP1) (Tejada-Jiménez et al., 2015). Immunolocalization studies indicate that MtNramp1 is mainly targeted to the plasma membrane. A loss-of-function nramp1 mutant exhibited reduced growth compared with the wild type under symbiotic conditions, but not when fertilized with mineral nitrogen. The mutant nodules have low nitrogenase activity. Exogenous iron and expression of wild-type MtNramp1 in mutant nodules have increased nitrogen fixation to normal levels. These data are consistent with a model in which MtNramp1 is the main transporter responsible for apoplastic iron uptake by rhizobia-infected cells in zone II of the root nodule. Among the seven identified members of the *M. truncatula* Nramp family, MtNramp1 has shown the highest expression level in nodules. Yeast complementation assays indicated that MtNramp1 can transport iron and manganese into cells, which is consistent with a putative role in iron uptake by nodule cells infected with rhizobia. However, for this to be the case in *M. truncatula*, it is puzzling that MtNramp1 is not found in the plasma membrane of the cells from zone II (Fig. 1).

The plant vacuole is the organelle involved in the maintenance of iron homeostasis in the cell. The homologs of tonoplast-localized Vacuolar Iron Transporter1 of *Lotus yaponicum* (SEN1) and *Glycine max* (GmVTL1a and GmVTL1b) are essential for nitrogen fixation (Hakoyama et al., 2012; Brear et al., 2020). Expression of SEN1 gene was detected exclusively in nodule-infected cells and increased during nodule development. The expression analysis has revealed a low expression of Nif gene in the nodules of sen1 mutant nodules compared with wild type nodules, which means the mutation affects nitrogen fixation activity. The differentiation of symbiosomes was impaired at a very early stage of nodule development (Hakoyama et al., 2012). The soybean has two homologs of this gene, GmVTL1a and GmVTL1b (Brear et al., 2020).The methods of yeast complementation, real-time PCR and proteomics were used by Brear et al. (2020) to prove that these genes are the putative soybean iron transporters. Brear et al. (2020) have characterized GmVTL1a functionality using complementation in plant mutants, hairy root transformation and microscopy. The expression analysis of GmVTL1a in nodules using a promoter-GUS fusion has shown that the expression has been detected in large infected cells of the inner cortex and in cells surrounding the vasculature, but not in freshly infected cells. A lower level of expression was detected in the smaller uninfected cells, cells of the inner cortex and a layer of cells in the outer cortex, but was absent in the root. GmVTL1a was co-localized with the endocytotic stain FM4-64 on the tonoplast and was also localized on the symbiosome membrane, similar to vacuole-residing proteins in the nodules of *M. truncatula*, by the mechanism of retargeting that is probably shared (Limpens et al., 2009; Gavrin, 2014). (Fig. 1).

Iron solubility and transport within and between plant tissues is facilitated by organic chelators, such as nicotianamine and citrate. Krivoruchko et al. (2018) characterized the nodule-specific citrate transporter of the multidrug and toxic compound extrusion family, *MtMATE67* of *M. truncatula.* The *MtMATE67* gene was induced early during nodule development and expressed primarily in the invasion zone of mature nodules. The MtMATE67 protein was located in the plasma membrane of nodule cells and also has been found in the symbiosome membrane surrounding bacteroids. In oocytes, MtMATE67 transported citrate out of cells in an iron-activated manner. Loss of MtMATE67 gene function resulted in accumulation of iron in the apoplast of nodule cells and a substantial decrease in symbiotic nitrogen fixation and plant growth. Taken together, the results point to a primary role of MtMATE67 in citrate efflux from nodule cells in response to an iron signal. This efflux is necessary to ensure the solubility of iron in $Fe3^+$ form and its mobility in the apoplast and uptake into nodule cells. Likewise, MtMATE67-mediated citrate transport into the symbiosome space would increase the solubility and availability of $Fe3^+$ for symbiosomes (Kryvoruchko et al., 2018) (Fig. 1).

Zinc is the cofactor of superoxide dismutases, which is an essential component of the cell's defense against reactive oxygen species (ROS). Zinc is essential for zinc-finger motif transcription factor (Rubio et al., 2007; González-Guerrero et al., 2014). As has been shown in the work of Abreu et al. (2017), zinc transporter MtZIP6 (Zinc-IronPermease6) is located in the plasma membrane of infected cells. MtZIP6 is responsible for zinc uptake from the apoplast and has a crucial role for root nodule nitrogen fixation. The silencing of MtZIP6 by method of RNA interference negatively affected the level of nitrogen fixation in silenced nodules (Abreu et al., 2017). (Fig. 1).

Calcium. The symbiotic signaling pathway starts with the oscillations of calcium ion concentration (Ca^{2+}) in the nucleus of root cells (Oldroyd, 2013). Nod factor-induced Ca^{2+} oscillations have been observed in *M. truncatula* (Wais et al., 2000), *Pisum sativum* (Walker et al., 2000), *Phaseolus vulgaris* (Cardenas et al., 1999) and *Lotus japonicus* (Harris et al., 2003). In a recent study, Granqvist et al. (2015) analyzed calcium responses in a variety of legumes and also in the nodulating non-legumes *Parasponia andersonii* and *Alnus glutinosa.*

Granqvist et al. (2015) concluded that Ca^{2+} oscillations are a common feature of bacterial recognition within the nitrogen fixing clade and a conserved feature of bacterial recognition in all nodulating species analyzed. However, detailed information about the pathways of calcium transport to the infected cell of the root nodule and symbiosomes and putative carriers is limited. (Fig. 1).

Transport of metal ions by rhizobia. For information concerning the transport of metal ions by rhizobia, we would like to refer to the comprehensive review of Abreu et al. (2019). They described the data concerning metal ion transport in rhizobia *ex-planta* and partly the transport of ions most abundant in symbiosomes. Several families of transporters participating in the efflux/influx of metal ions in rhizobia have been identified, though only few of them were found to be indispensable for the process of nitrogen fixation. These results show the complexity of ion membrane transport in infected cells. Bacteroids are completely dependent on the nutrients delivered by the plant. Hence, rhizobia performance in the nodules is quite different from growth conditions *ex planta* (Abreu et al., 2019).

In silico gene expression analysis of potassium/sodium ion transporters, channels and exchangers

Potassium is not one of the metalloenzymes involved in the process of nitrogen fixation like nitrogenase or in the maintenance of oxygen levels like leggemoglobin. However, potassium is functional in osmoregulation, membrane transport and anion neutralization, as well as in the control of cytoplasmic and luminal pH in endosomes, regulation of membrane potential, and enzyme activity (Wang and Wu, 2013; Ragel et al., 2019). These are the functions that are crucial also for the root nodule and infected cell. Potassium (K^+) is an essential macronutrient in plants. Potassium deficiency significantly reduces the potential for plant growth and development. By contrast, sodium (Na^+), while beneficial to some extent in the maintenance of cell turgor, at high concentrations disturbs and inhibits various physiological processes and plant growth. Some functions of K^+ can be undertaken by Na^+ but K^+ homeostasis is severely affected by salt stress (Adams and Shin, 2014). Maintenance of intracellular K^+/Na^+ homeostasis is a crucial mechanism for plant growth and development.

It is a well-known fact that root nodules are more sensitive to stress caused by agents such as salt and heavy metals, than the roots of the host plant and the plant itself. Salt and heavy metals are detrimental for nodule formation and nitrogen fixing activity (Zahran, 1999; Tsyganov et al., 2007; Shvaleva et al., 2010; Coba de la Peña and Pueyo, 2012; Brear et al., 2013; Bertrand et al., 2016). It is quite possible that such a high vulnerability of the nodules is caused by spatial changes in K^+/Na^+ ion homeostasis of infected tissue. With the aim of improving nodule stress tolerance, different works for selection and bio-engineering of host plants (Tsyganov et al., 2007, 2020; Coba de la Peña et al., 2010; Belimov et al., 2015) and microsymbionts (Domínguez-Fererras et al., 2009; Nonnoi et al. 2012; Quiñones et al. 2013) have been performed. Proteomics and transcriptomics techniques have been used to detect genes and proteins involved in stress tolerance of root nodules (Lu et al., 2017; Baig et al., 2018).

However, a comprehensive model of K^+ and Na^+ transport and homeostasis in infected cells of the root nodule has not yet been developed.

Most of the current knowledge concerning ion transport of K^+ and Na^+ in plants has been obtained from studies using *Arabidopsis thaliana* as a model. The main families of K^+-permeable transport systems include the families of HAK-KUP-KT transporters, Shaker-like K^+ channels, HKT transporters, and cation-proton antiporters (CPA). The transport of Na^+ is mainly dependent on the transporters from the group of cation/proton exchangers/antiporters (Sze et al., 2018; Ragel et al., 2019; Villette et al., 2020).

Plant HAK-KUP-KT proteins. The transporters of the HAK-KUP-KT family are highly selective for K^+ and are crucial for organisms facing external solutions containing very low K^+ concentrations (μM range). These transporters possess 10 to 14 transmembrane domains with both N- and C-termini at the membrane intracellular side. Members of this family have been widely associated with high-affinity K^+ uptake from the soil, and other roles related, for example, to K^+ translocation, control of water movement at the plant level, salt tolerance, osmotic/drought responses, transport of other alkali cations, and developmental processes in plants, such as root hair growth and auxin distribution (Li et al., 2018; Santa-Maria et al., 2018). These diverse functions of KT/HAK/KUP transporters may all result from their critical roles in cellular K^+ homeostasis (Ragel et al., 2019).

Shaker channels are involved in a K^+ constant transport across the plasma membranes. Functional channels are tetrameric proteins arranged around a central pore and are composed by assembling four Shaker subunits encoded either by the same gene (homomeric channel) or by different genes (heteromeric channel) (Dreyer et al., 2004; Sharma et al.; 2013; Véry et al., 2014). Approximately 80 % of high- and low-affinity K^+ uptake in Arabidopsis can be attributed to the sum of functions of AtAKT1 and AtHAK5 (a member of the HAK/KUP/KT family of transporters (Coskun and Kronzucker, 2013). The Shaker channel GORK, the outward K^+ channel, controls the closure of stomata in Arabidopsis. The opening of stomata are controlled by inward Shaker channels: KAT1, KAT2, and AKT1 (Lebaudy et al., 2008). These channels are also involved in phloem K^+ loading and unloading. In *Vitis vi-*

Table 1. Transporters/channels/exchangers of potassium and sodium of *Arabidopsis thaliana* and *Medicago truncatula* homologs

***Arabidopsis thaliana* protein/gene names in UniProt/ homolog of *Medicago truncatula* protein/gene names from Symbimics database**	
GROUP 1	**GROUP 2**
Potassium transporter 2 (POT2 KT2) **/** Potassium transporter 2 (Medtr3g094090.1)	**Potassium transporter 3 (POT3** KT3**)** / Potassium transporter 3 (Medtr8g099090)
Potassium transporter 5 (POT5 HAK5**) /** Potassium transporter 5 (Medtr4g099260.1)	**Potassium transporter 6 (POT6** HAK6) / Potassium transporter 6 (Medtr5g034500.1)
Potassium transporter 8 (POT8 HAK8**)** / Potassium transporter 8 (Medtr6g007697.1)	**Potassium transporter 7 (POT7** HAK7**) /** Potassium transporter 7 (Medtr2g008820.1)
Potassium channel AKT1(AKT1 At2g26650**) /** Potassium channel AKT1 (Medtr4g113530.1)	**Potassium channel GORK** (GORK **At5g37500) /** Potassium channel SKOR (Medtr5g077770.1)
Potassium channel AKT2/3 (AKT2 AKT3**)** / Potassium channel AKT2/3 (Medtr2g006870.1)	**Sodium/hydrogen exchanger 2 (NHX2** At3g05030**) /** Sodium/ hydrogen exchanger 2 (Medtr1g081900.1)
Potassium channel KAT1 (KAT1 At5g46240**)**/ Potassium channel KAT1 X1 (Medtr8g446430.1)	**Sodium/hydrogen exchanger 6 (NHX6** At1g79610**) /** Sodium/ hydrogen exchanger 6 (Medtr2g028230.1)
Potassium channel KAT3 (KAT3 AKT4**)** / Potassium channel KAT3 (Medtr3g108320.1)	**Cation/H(+) antiporter 21** (**CHX21** At2g31910) / Cation/H(+) antiporter 15 (Medtr5g009770)
Sodium/hydrogen exchanger 7 (NHX7 SOS1**) /** Putative potassium channel, voltage-dependent, homolog of **Sodium/hydrogen exchanger 7/**ERG, cation/H+ exchanger (Medtr2g038400.1)	**Cation/H(+) antiporter 17(CHX17** At4g23700**)** / Cation/H(+) antiporter 18 (Medtr5g009770.4)
Sodium transporter HKT1 (HKT1 At4g10310**) /** Probable cation transporter HKT6 **(**Medtr6g092840.1)	
Sodium/calcium exchanger NCL (**NCL** At1g53210) / Sodium/calcium exchanger NCL (Medtr2g078240.1)	

nifera the homolog of AKT2 VvK3.1 channel mediates K^+ unloading in the berries and also regulates the transmembrane K^+ gradients of phloem cells (Villette et al., 2020). SKOR Shaker-like K^+ outward rectifying channel in Arabidopsis mediates long-distance K^+ transport from roots to shoots, and it is a plasma membrane Na^+/H^+ antiporter (Demidchik, 2018).

Cation/H^+ antiporters (CPA). The group of $K^+(Na^+)/H^+$ exchangers (NHX) is a sub-group of CPA (Sze and Chanroj, 2018). Exclusion of sodium ions is one of the most important traits for salt tolerance in plants. Plant NHX antiporters are membrane proteins that transport protons (H^+) across a membrane in exchange for Na^+ or K^+. Studies show that plant NHX antiporters are involved in the maintenance of cellular ion homeostasis and pH regulation, and play significant roles in diverse cellular processes, Na^+ and K^+ movement, vesicle trafficking and fusion, growth and development, and salt tolerance (Wu et al., 2016). In Arabidopsis these transporters are localized in different membrane compartments: SOS1/AtNHX7 on the plasma membrane, AtNHX5/6 on endosomes, and AtNHX1–4 on the tonoplast (Chanroi et al., 2012). The mutations in the gene NHX7/SOS1 cause oversensitivity to salt due to disrupted efflux of sodium (Sze et al., 2004; Pardo et al., 2006, Bassil and Blumwald, 2014; Sandhu et al., 2018). Upregulation of NHX7/SOS1 was shown to be associated with salt tolerance in Medicago species (Liu et al., 2015; Sandhu et al., 2017). NHX1 and NHX2, localized in the tonoplast, are the two major NHX isoforms. These antiporters sequester Na^+ in vacuoles, which helps to maintain Na^+/ K^+ homeostasis (Bassil et al., 2011; Sandhu et al., 2018). NHX1 and NHX2 are essential for active K^+ uptake at the tonoplast and for turgor regulation (Barragan et al., 2012).

Data concerning K^+ and Na^+ transport in nodules are scarce (Benedito et al., 2010; Udvardi and Poole, 2013; Drain et al., 2020) and an integral picture of the transporters in infected cells is not yet available. With the aim of obtaining more information on K^+ and Na^+ transport in the root nodule we have performed *in silico* gene expression analysis of *M. truncatula* K^+ and Na^+ transporters/channels/exchangers using the database Symbimix, obtained by Laser-capture microdissection of root nodule zones (Roux et al., 2014). The genes of K^+ and Na^+ transporters/channels/exchangers of *A. thaliana* were identified in the available genomic and cDNA sequence databases (https://www.uniprot.org). Selected protein sequences have been used for the search for *M. truncatula* homologs in public bioinformatic resources (https://phytozome.jgi.doe.gov/pz/portal and https://www.ncbi.nlm.nih.gov). The list of *M. truncatula* homologs and Arabidopsis genes is presented in Table 1.

Gene / Nodule zones	Transporter							Channel					Exchanger					
	KT2	KT3	HAK5	HAK6	HAK7	HAK8	HKT6	AKT1	AKT2/3	KAT1	KAT3	SKOR	NHX2	NHX6	CHX15	CHX18	NHX7-like	NCL
F1	65	22	50	3	15	58	60	60	38	39	47	12	15	10	13	14	40	22
FIId	17	19	10	5	16	21	35	5	28	32	20	9	15	23	12	14	41	25
FIIp	7	22	29	10	22	14	5	5	18	17	5	10	23	24	19	19	19	26
IZ	7	19	12	30	20	3		18		12	8	45	16	23	30	30		15
ZIII	6	17		50	28	6		15	12		22	25	32	24	26	26		12

high
medium
low
absent

Fig. 2. Potassium and sodium channels, transporters, and exchangers transcripts expression in developmental zones of root nodule (derived from *Symbimics* database).
A more detailed description of the figure is given in the text.

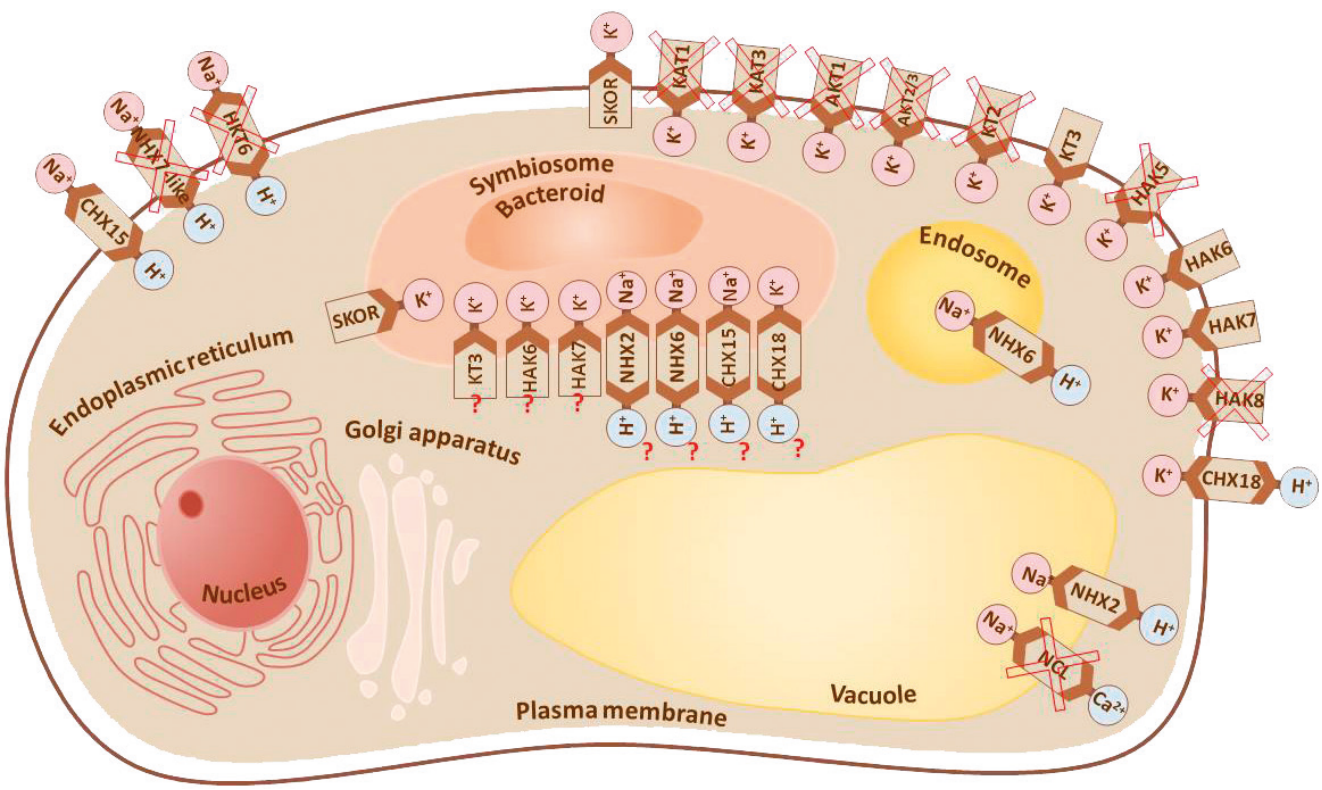

Fig. 3. The putative pattern of location of K^+/Na^+ transporters in a mature infected cell.
AKT1, Potassium channel; **AKT2/3**, Potassium channel; **CHX15**, Cation/H+ exchanger 15; **CHX17**, Cation/H+ exchanger 17; **CHX18,** Cation/H+ exchanger 18; **HAK5**, Sodium transporter 5; **HAK6**, Sodium transporter 6; **HAK7**, Sodium transporter 7; **HAK8**, Sodium transporter 8; **KAT1**, Potassium channel; **KAT3**, Potassium channel; **KT2/KUP2**, Potassium transporter; **KT3/KUP4** (Tiny root hair 1 protein), Potassium transporter; **NCL**, Sodium/calcium exchanger; **NHX2**, Sodium/hydrogen exchanger 2; **NHX6**, Sodium/hydrogen exchanger 6; **NHX7**, Sodium/hydrogen exchanger 7; outward-rectifying potassium channel **SKOR**.

The levels of expression *in silico* of putative *M. truncatula* transporters/channels/exchangers have been estimated in nodule developmental zones according to Roux et al. (2014). These are: the nodule meristematic zone, termed as fraction I (FI); the region below FI, collected as a distal and a proximal fraction (FIId and FIIp), and corresponding to ZII cells undergoing differentiation or infection; the interzone II–III (IZ), which separates

ZII from the nitrogen fixation zone ZIII (Vasse et al., 1990; Roux et al., 2014) (Table 1, Fig. 2). For group 1 in Table 1, transporters with low or absent expression in the nitrogen fixation zone (ZIII) were selected. In group 2, the transporters with high stable expression level or upregulated in nitrogen fixation zone were excerpted. (Fig. 2). According to the expression analysis, all selected transporters, channels and exchangers showed expression in the meristematic and rapidly expanding cell layers of post-meristematic cells and young infected cells (F1, F2d, F2p, IZ) (Fig. 2). However, in the nitrogen fixation zone (ZIII) the expression was maintained only by a fraction of these genes.

The putative pattern of transporters/channels/exchangers' location in infected cell is presented in Figure 3. The transporters with a high level of expression in the zone of active nitrogen fixation (ZIII) were selected as candidate genes for the transporters in mature infected cells. The genes that showed high expression in the meristem of the nodule and young post-meristematic cells, but low or absent expression in ZIII, we consider to be not involved in the transport of K^+ and Na^+ in the zone of nitrogen fixation. In Figure 3 these genes are marked by crosses. To date, only the expression (Drain et al., 2020) and localization in the symbiosome membrane of Shaker-like K^+ outward rectifying channel SKOR/GORK, coded by a gene upregulated in IZ and ZIII, is confirmed (Fedorova et al., 2021). For the localization and expression of other genes experimental proof is still needed, so they are labelled with a question mark (Fig. 3).

For now, we cannot indicate the reasons for the differential expression of the transporters in ZIII. We also have no data to specify whether the level of expression of some genes coding for K^+ transport in mature infected cells is sufficient to meet the need for potassium of the infected cell and bacteria housed in it. However, some of the genes encoding Arabidopsis K^+ transporters, such as CHX17, HAK5 and KEA5, have been shown to be induced by K^+ deficiency (Adams and Shin, 2014). We can guess that an infected cell may experience limitations in K^+ in comparison with non-infected cells situated in the apical cell layers of the nodule that display a high level of expression of these transporters.

It is also quite interesting that exchangers/antiporters involved in the balance of sodium in the root nodule show substantial expression in the root nodule (Table 1). The expression of Sodium/hydrogen exchanger 2 (NHX2) (Medtr1g081900.1), and Sodium/hydrogen exchanger 6 (NHX6) (Medtr2g028230.1) is upregulated in ZIII. This would be the expected outcome in case of applied salt, but it is puzzling why such an event is observed in untreated nodules. It is possible that the exchangers/antiporters coded by these genes could be involved in translocation of other ions required by infected cells, partly substituting the K^+ transporters coded by downregulated genes or the genes that stop being expressed in infected cells. The possibility that some portion of sodium ions may occasionally go through other ion channels, due to the similarity to potassium ions in size and charge, also cannot be excluded.

However, several genes, homologs of Arabidopsis genes involved in the $K^+/Na^+/H^+$ exchange and salt stress defense reaction, are not expressed in ZIII. These are the *M. truncatula* homolog (Medtr6g092840.1) of sodium transporter HKT1 (HKT1 At4g10310), which plays a central role in plant tolerance to salt (Rus et al., 2003) by translocation of Na^+ from the roots to the transpiring leaves, the *M. truncatula* homolog of NHX7/AtSOS1 (Medtr2g038400.1), a main player in salt stress response, regulating the secretion of Na^+ from the cytoplasm to the extracellular space (Qiu et al., 2003), and the sodium/calcium exchanger NCL (Medtr2g078240.1) involved in Na^+/Ca^{2+} homeostasis in stress conditions. It is interesting that these three genes code the plasma-membrane-located proteins functional in translocation of Na^+ across the plasma membrane. Due to this, the means for Na^+ detoxication via the plasma membrane in the zone of nitrogen fixation may become unavailable because Na^+ removal from the host cell cytoplasm is limited to the action of the exchangers located in the tonoplast and endosomes: Sodium/hydrogen exchanger 2 (Medtr1g081900.1) and Sodium/hydrogen exchanger 6 (Medtr2g028230.1). By analogy to Arabidopsis's homologs, these exchangers have to be functional in sequestering Na^+ in vacuoles and endosomes (Bassil et al., 2019). Vacuolar ion transport kinetics, including estimates of apparent Km for K^+ and Na^+, indicated that these exchangers also mediate K^+ transport to the vacuoles in Arabidopsis, where NHX1, NHX2, and NHX4 are the main transporters mediating vacuolar K^+ uptake (Bassil et al., 2019). However, it is quite possible that the vacuolar exchanger MtNHX2 would have a low functionality in infected cells of *M. truncatula* due to the changing of vacuolar lumen pH in mature infected cells. The vacuoles of mature infected cells lose the acidic pH and become neutral or slightly basic (Gavrin et al., 2014). Therefore, it would be functional only in non-infected cells of the zone of nitrogen fixation. The above-described features of infected cells may partly explain the incapacity of these cells to manage salt stress. However, these hypothetical assumptions need further experimental verification. Concluding this part of the minireview we would like to point out that, as is shown by the *in silico* analysis presented above, the expression analysis that is routinely made on RNA extracted from the integral nodule does not accurately reflect the differences between different developmental zones of the nodule and between infected and non-infected cells. We believe that to obtain information concerning the real situation, a localization study on the cellular level is

crucial. Research in this area is timely and can provide much needed information for improving the stress resistance of root nodules and increasing the duration of their functional activity.

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